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Hydrogen Peroxide has been shown to be efficiently electroreduced at an electrode modified with a hydrophilic, permeable film of horseradish peroxidase covalently bound to a 3-dimensional epoxy network having polyvinyl pyridine (PVP)-complexed $[Os(bpy)_2Cl]^{+2/+3}$ redox centers.[1] Four peroxide sensing cathodes based on peroxidases from Arthromyces ramosus, horseradish and bovine milk are compared. Their sensitivity at 0.0V (SCE) ranges from 0.1 - 1.0 A cm^{-2} M^{-1}, and their limiting currents relate to the enzyme's ability to complex with the redox epoxy network.

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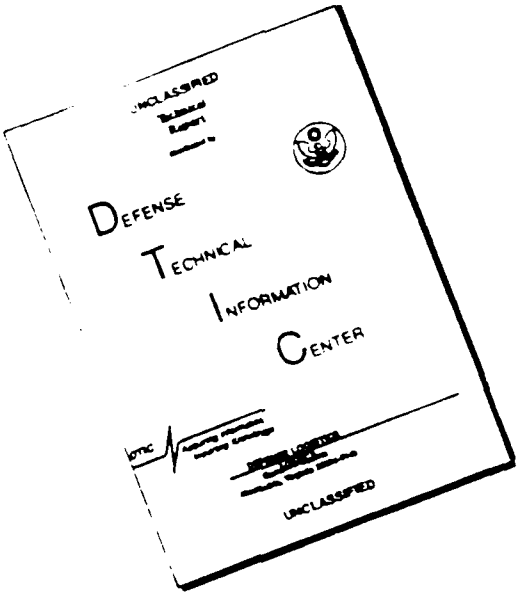

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Hydrogen Peroxide Electrodes Based on Electrical Connection of Redox Centers of Various Peroxidases to Electrodes through a Three-Dimensional Electron Relaying Polymer Network.

Mark S. Vreeke and Adam Heller

Abstract

Hydrogen Peroxide has been shown to be efficiently electroreduced at an electrode modified with a hydrophilic, permeable film of horseradish peroxidase covalently bound to a 3-dimensional epoxy network having polyvinyl pyridine (PVP)-complexed $[Os(bpy)_2Cl]^{+2/+3}$ redox centers.[1] Four peroxide sensing cathodes based on peroxidases from Arthromyces ramosus, horseradish and bovine milk are compared. Their sensitivity at 0.0V (SCE) ranges from 0.1 - 1.0 A cm^{-2} M^{-1}, and their limiting currents relate to the enzyme's ability to complex with the redox epoxy network.

Hydrogen Peroxide Detection

Electrochemical and optical hydrogen peroxide detection forms the basis for several medical diagnostic tests. Electrochemical detection offers the advantages of smaller required sample size and ease of integration into a flow system. A common electrochemical scheme uses an oxidase to catalyze the selective translation of a substrate concentration to an H_2O_2 concentration. This translation is followed by amperometric assay of the H_2O_2, e.g. by its oxidation on platinum at 700mV (SCE). At 700mV (SCE) electrooxidation of various reducing species in the biological samples can interfere with the assay.

Peroxidase enzymes (POD) catalyze the reduction of H_2O_2 by electron donors (HA) in the following reaction

$$2HA + H_2O_2 \xrightarrow{(POD)} 2H_2O + 2A \qquad (1)$$

Amperometric peroxidase based H_2O_2 sensors have been made by using fast reversible redox couples (see Tables I and II). In these, the reducing member of donates electrons to H_2O_2 , is oxidized, and is then cathodicly reduced.

Horseradish peroxidase (HRP) is the most commonly used peroxidase for diagnostic testing (Table I). Other peroxidases (Table II) are used less frequently because they are less easily available or cost more. Tables I and II show the detection schemes vary in their method of immobilization of mediator and enzyme. At one extreme one finds systems based on the direct transfer of electrons from the electrode surface through surface bound mediators to HRP redox centers contacting the surface. At the other extreme one finds systems with freely diffusing mediators and enzyme.

H_2O_2 Detection based on 3-Dimensional Redox Epoxy Networks

Here we describe electrodes based on peroxidases from horseradish (HRP), Arthromyces ramosus (ARP), and bovine milk (LOP) immobilized in a three dimensional redox epoxy hydrogel on a current collector. In the case of HRP we used either the purified native enzyme or its sodium periodate oxidized derivative (HRPox). The redox hydrogel was made of a poly(4-vinyl pyridine) backbone partially complexed by osmium bipyridine redox centers are electron donors (species HA in reaction 1) and relay electrons from the electrode to the reactive centers of the peroxidase (Figure 1). The ethyl amine groups enable the cross linking of the enzyme containing polymer film with a polyethylene glycol diglycidyl ether (reaction 2).

(2)

The three dimensional redox epoxy offers some of the advantages of both the freely diffusion systems and the immobilized systems. As in freely diffusing mediator based systems, not only the electrode adsorbed enzyme molecules, but also those in the redox polymer containing volume are electroactive. At the same time, there is no need to add mediator to the sample, and the mediator can not leach out or contaminate the sample.

POD enzymes are rather small (40 ≈ kd), and their active groups are positioned relatively close to the enzyme surface. This allows direct electron transfer between the POD enzymes and the electrodes. Figure 2 shows the dependence of the current on potential for HRP immobilized in a hydrogel similar to the electron relaying one but without the electron relaying osmium redox sites. Here only those enzyme molecules actually adsorbed on the electrode surface in electrical contact with it contribute to the signal. In contrast, when the enzyme is immobilized in the hydrogel with electron relaying osmium centers, there is a

hundred fold increase in current because enzyme molecules contacting relays are also "wired". (Figure 3)

Peroxidase Sensor Response

The dependence of the current response on H_2O_2 concentration for optimized HRP, HRPox, ARP, and LOP cathodes is shown in figure 4. The ARP and HRPox cathodes show a linear range from 0.1 to 100μM H_2O_2. The limit of detection for HRPox is 10nM. The cathode is slightly less sensitive then the HRPox cathode, however it has the advantage of increased dynamic range. The LOP cathode does not exhibit the good sensor response of the other enzymes. Its linear range is narrow, and sensor deteriorates more rapidly than the HRP sensor, that shows only a 10% loss over the course of 3 days continuous operation. The LOP sensor looses 10% of its output in hours.

The weight fraction of POD in the redox epoxy film effects the sensor response. As the amount of enzyme is initially increased in the film the current increases, reaching a maximum at 8 to 50% weight POD. As more enzyme is added current then decreases. The shape of the curve reflects the fact that at low enzyme loading the sensor is limited by the number of catalytic sites. However, as the fraction of electrically insulating POD increases the sensor becomes electron transport limited, and as more enzyme is added the current decreases.

Figures 5 to 8 show this effect. The ARP (figure 5), LOP (figure 6) and HRP (figure 7) obtain their respective maximum currents at 20 to 45%, 35 to 50% and 20 to 40% enzyme loading. These percentages are similar to the results obtained for anodes using the same redox polymer and glucose oxidase.[21] It is interesting to note the variation in current maximum for the two HRP enzymes. For $NaIO_4$ oxidized HRP the current maximum is found at 8-20% enzyme loading

(figure 8) vs. 20-40% enzyme loading for native HRP (figure 7). $NaIO_4$ treatment of the glycoprotein is a standard procedure for generation of aldehydes by the oxidation of sugar residues. The aldehydes produced can be covalently bound to the redox polymer, which is a polyamine, in a reaction where multiple Shiff bases are formed. Formation of a dense system of covalent bonds implies tight binding of the enzyme and its "wiring" redox polymer. It results in effective electrical connection of a large fraction, perhaps most, of the enzyme molecules present. Thus the current rises rapidly and to a high level as enzyme is added, then becomes limited by the network's current carrying capacity when the fraction of insulating enzyme becomes excessive.

Electrostatic Interaction of Polymer and Enzyme

We account for the differences between the sensors by the different electrostatic interactions between the polymer and enzyme. Strong electrostatic interaction between the enzyme and the redox polymer is expected to lead to tight coupling of the enzyme and the "wiring" polymer, and thus to a shorter average distance for electron transfer. Because the redox polymer is a polycation, the greater the negative charge of the enzyme at neutral pH, the higher the current. This explanation is consistent with the interpretation of the behavior of the response of different FAD enzyme sensors made with the present redox polymer.[22] The formation of polymer-enzyme complexes is readily observed in isoelectric focusing (IEF) experiments. Figure 9 shows IEF runs for the 4 enzymes. ARP, HRP and LOP are respectively negatively, slightly positively and positively charged at pH 7. Native HRP focuses as two separate isoenzymes with very close PI's. The HRPox forms a complex mixture, and does not focus to a single spot, but is more negative than HRP.

Comparison of the results of the isoelectric focusing experiments with the limiting currents supports the proposition that a positive electrostatic interaction contributes to sensor performance: the order of increasing negative charge LOP<HRP<HRPox<ARP parallels the increase in limiting currents.

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(22) Work in progress.

Table I: Amperometric H_2O_2 Sensors Based on HRP Modified Electrodes

Electrode Surface	Mediator or Redox Matrix	Electrode Potential[a]	Sensitivity $Acm^{-2}M^{-1}$	Linear range μM	Comments	Reference
Glassy carbon	None	0.0	10^{-2}	---	HRP covalently bound to a hydrophilic epoxy network. Polyvinyl pyridine-derived polyamine crosslinked with PEGDE	1
Glassy carbon	Polymer 1	0.0	1	0.1-100	HRP covalently bound to hydrophilic epoxy network. Polymer 1 crossinked with PEGDE	1
Spectrographic graphite	None[c]	0.05	0.175	0.1-500	BSA with glutaraldehyde cross-linking	2
Carbon paste	O phenylene-diamine[d]	0.15	N.A.[b]	3.1-200	butanone peroxide was used as the substrate	3

Pt	Hexacyanoferrate (.01M)[d]	-0.05	Note e	5-1700	HRP was immobilized onto a nylon net	4
HMP[f] TCNQ	None[d]	0.05	.168	----	HRP entrapped with dialysis membrane	5
SnO_2	ferrocene carboxylic acid[d]	0.2	.01	0.01-1	HRP immobilized with glutaraldehyde	6
Carbon paste	ferrocene[d]	0.05	N.A.[b]	0.1-10	Nafion coating was applied to the electrode to prevent loss of mediator	7
Graphite foil	potassium hexacyanoferrate(II)[d]	0.02	0.03	-600	electrolyte was dioxane with 15% aqueous buffer	8
Carbon fiber[g]	None	Note h		10-5000	The biotin-avidin complex was used to obtain a surface layer of HRP	9

Pt, organic metal, or glassy carbon	potassium ferrocyanide	Note i	-----	-----	membrane with albumin and glutaraldehyde	10
Spectrographic graphite or Carbon film	hexacyano-ferrate (II)[d]	0.0	Note e	.1-1000	HRP immobilized on arylamino derivatized controlled-pore glass, packed into a flow through reactor	11
Amino silylated glassy carbon	hexacyano-ferrate (II)[d]	0.0	Note i	-----	Glycerophosphate oxidase, HRP and BSA were covalently cross-linked on the glassy carbon surface.	12
Glassy carbon	hexacyano-ferrate (II)[d]	0.0	Note i	-----	Albumin, glutaraldehyde, HRP and oxidase (xanthine, uricase, glucose) matrix held close to the electrode with a dialysis membrane.	13
Gold or graphite	Several[k]	Note k	2.0[l]	0.05-6[l]	HRP was free in solution	14

TableII: Amperometric H_2O_2 sensors utilizing biocatalysts other than HRP

Electrode Surface	Mediator or Redox Matrix	Electrode Potential[a]	Sensitivity $Acm^{-2}M^{-1}$	Linear range μM	Comments	Reference
Spectrographic graphite	None	0.1	1	.27-2.46	Fungal peroxidase from Arthromyces ramosus immodilized with a difunctional carbodiimide	15
Pyrolytic graphite edge	None[i]	-.55	0.12	-70	Cytochrome C peroxidase was used free in solution	16
Gold	1,3'-dimethyl ferrocene ethylamine[d]	-0.1	1.76	-----	cytochrome C peroxidase was immobilized on a nylon membrane	17
SnO_2	None	0.2	.0009	1-500	Heme nonapeptide (MW = 1600)	18

Edge oriented pyrolytic graphite	None	0.0	3.0	NA	Cytochrome C peroxidase (MW = 34.1kDa) was adsorbed onto the surface. Electrode polishing protocol was found to impact on the stability and reproducibility of the electrocatalytic response.	19
Gold	None	--	--	-----	The surface was modified with methyl viologen. HRP reduction below -.7v and oxidation above -.1 to -.6v[a]	20

a) potential vs SCE
b) macroporous electrode, the true surface area is unknown
c) uncertainty as to whether surface species created during electrode pretretment are mediating
d) freely diffusing mediator

e) flow system

f) probably mediated by soluble component of organic metal or reaction product of organic metal

g) microelectrode

h) cyclic voltametry used to provide selective detection of oxygen generated by autocatalytic decomposition of hydrogen peroxide

i) HRP incorporated into a bienzyme system

j) aminoglycosides gentamycin or neomycin were used it bind cytochrome C peroxidase and facilitate electron transfer

k) mediators used and redox potential are: [Ru(NH3)5py](ClO4)3 = +28 CpFeC2B9H11 = -80 1,1' dimethyl-3-(2-aminoethyl) ferrocene = +75 (2-aminoethyl)ferrocene = +185 Ferrocenemonocarboxylic acid = +275 aminomethylferrocene = +309mV

l) best reported result for ferrocene monocarboxylic acid

Figure Captions

Figure 1. Redox cycles occurring in the 3-dimensional redox epoxy hydrogel. POD represents any of the following enzymes: native horseradish peroxidase, $NaIO_4$ treated horseradish peroxidase, lactoperoxidase, or Arthromyces ramosus peroxidase.

Figure 2. Dependence of current on potential for a $NaIO_4$ oxidized horseradish peroxidase immobilized in a 3-dimensional epoxy hydrogel free of electron relaying osmium redox centers. (A) no H_2O_2; (B) 0.1mM H_2O_2 Conditions: aerated pH 7 physiological phosphate buffer solution: scan rate 2.5 mV s^{-1}; 500 RPM.

Figure 3. Electrode as in figure 2, but with osmium electron relaying redox centers. (A) no H_2O_2; (B) 0.1 mM H_2O_2; (C) 0.5 mM H_2O_2 Conditions for A and B are as in figure 2. C was done as 2000 RPM.

Figure 4. Dependence of current density on hydrogen peroxide concentration for cathodes based on different peroxidases. (open circles) $NaIO_4$ treated horseradish peroxidase; (closed circles) native horseradish peroxidase; (open squares) lactoperoxidase; (closed squares) Arthromyces ramosus. Each electrode contains approximately 10µg osmium redox polymer, 1µg polyethylene glycol diglycidyl ether crosslinker and 1 to 4µg peroxidase. Conditions: aerated pH 7 physiological phosphate buffer solution; 1000 RPM.

Figure 5. Dependence of current on the weight fraction of Arthromyces ramosus peroxidase (ARP) in the film. The osmium redox polymer and crosslinker amounts were held constant at approximately 10 and 1µg. Conditions: aerated pH 7 physiological phosphate buffer solution; 1000 RPM.

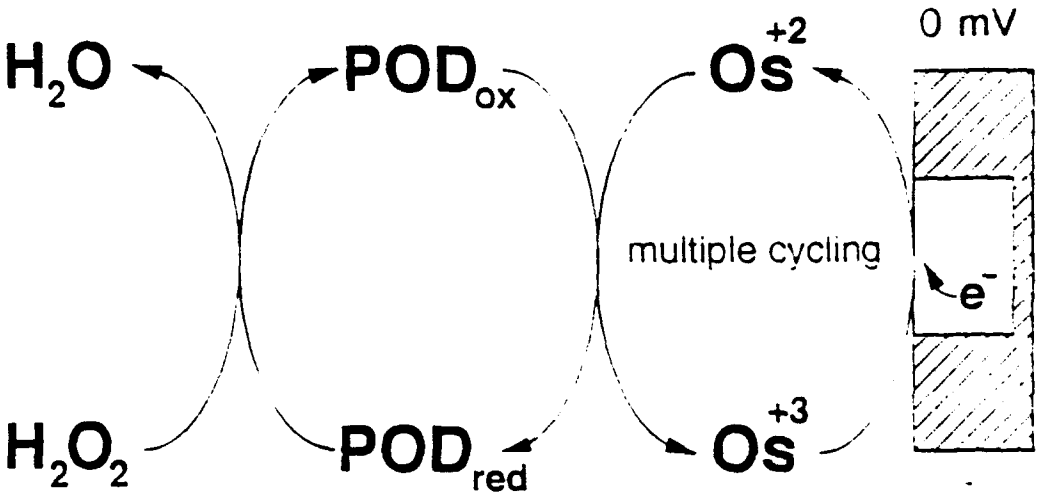
Figure 6. Dependence of current on the weight fraction of lactoperoxidase (LOP) in the film. The osmium redox polymer and crosslinker amounts were held

constant at approximately 10 and 1µg. Conditions: aerated pH 7 physiological phosphate buffer solution; 1000 RPM.

Figure 7. Dependence of current on the weight fraction of horseradish peroxidase (HRP) in the film. The osmium redox polymer and crosslinker amounts were held constant at approximately 10 and 1µg. Conditions: aerated pH 7 physiological phosphate buffer solution; 1000 RPM.

Figure 8. Dependence of current on the weight fraction of $NaIO_4$ treated horseradish peroxidase (HRPox) in the film. The osmium redox polymer and crosslinker amounts were held constant at approximately 10 and 1µg. Conditions: aerated pH 7 physiological phospnate buffer solution: 1000 RPM.

Figure 9. Isoelectric focusing of the 4 enzymes. The agrose gel was loaded with 3.5 to 9.5 pH ampholite to set up the gradient.



Three dimensional redox epoxy hydrogel

fig. 2

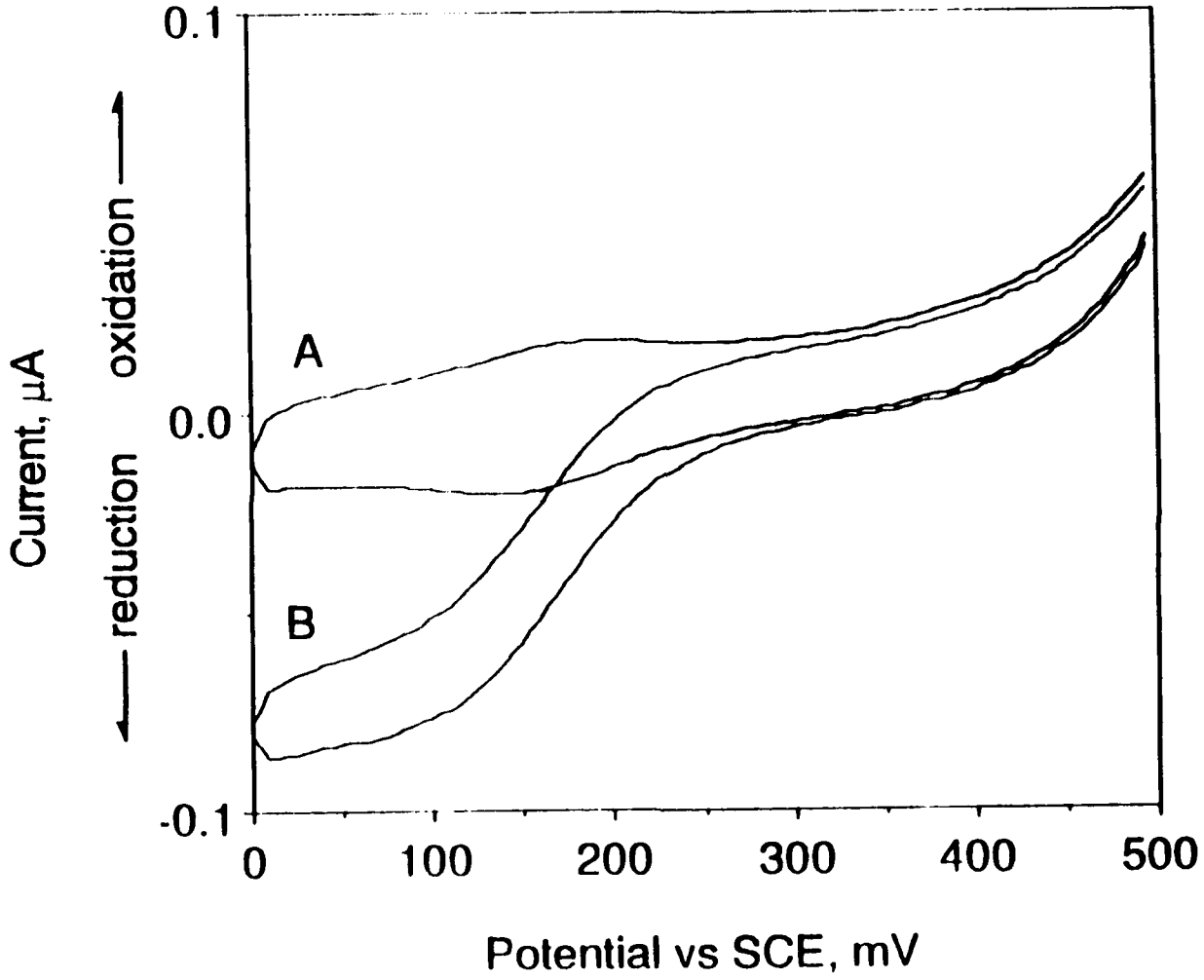

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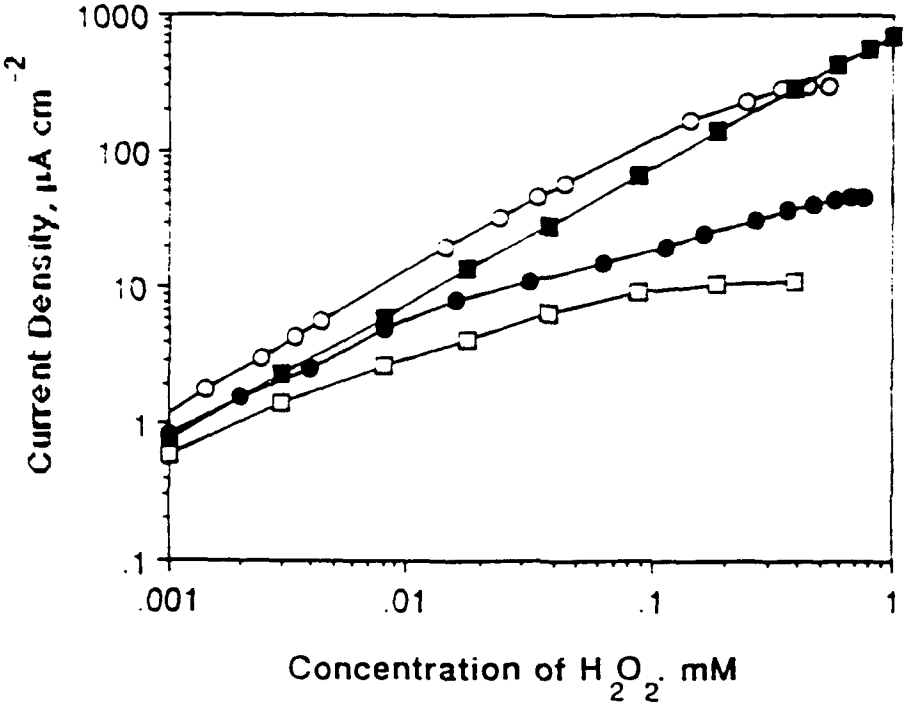
Fig. 3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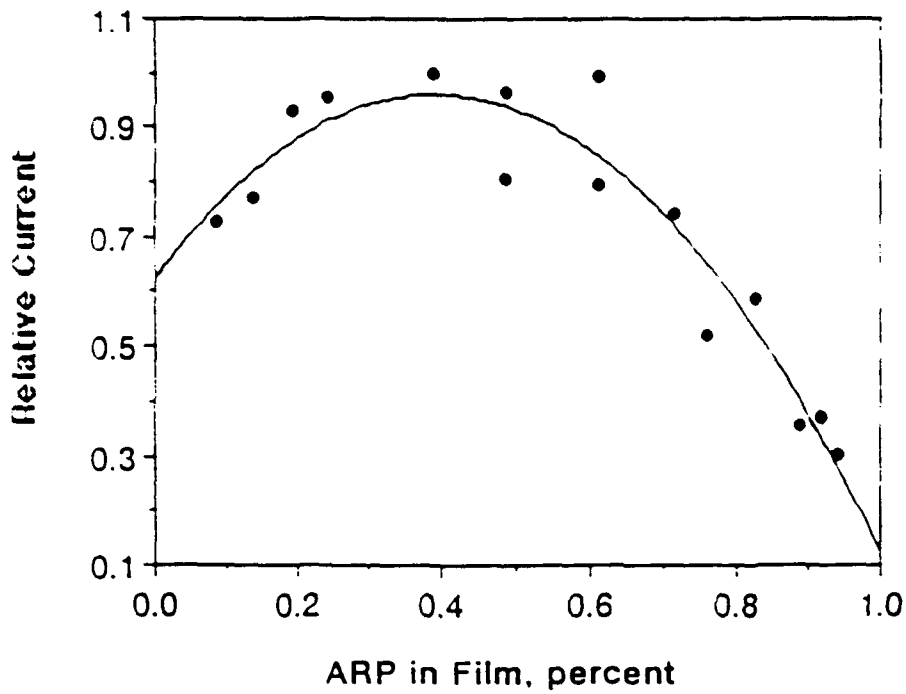
Concentration of H2O2, mM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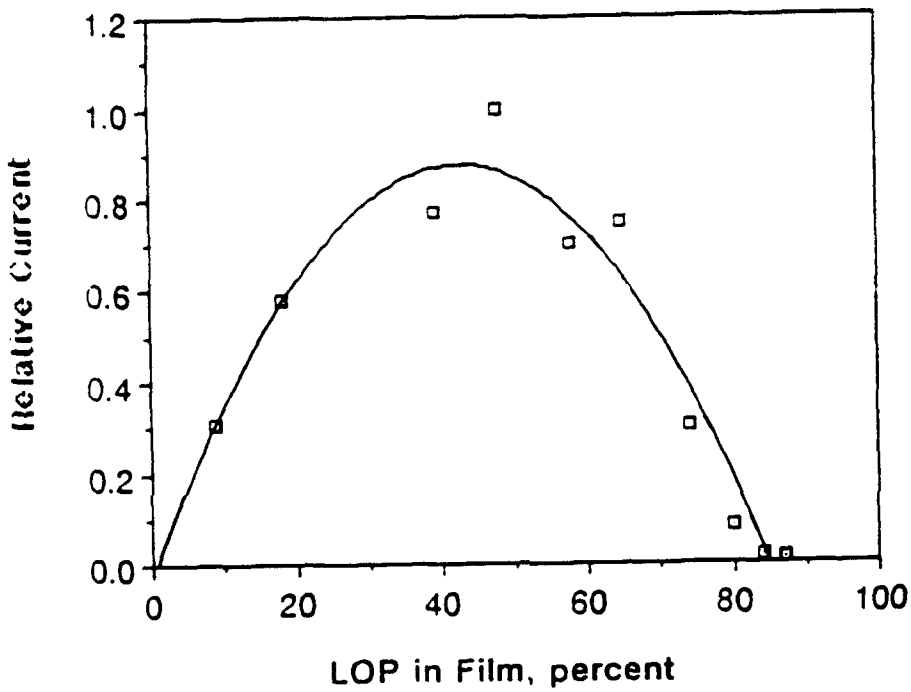
ARP in Film, percent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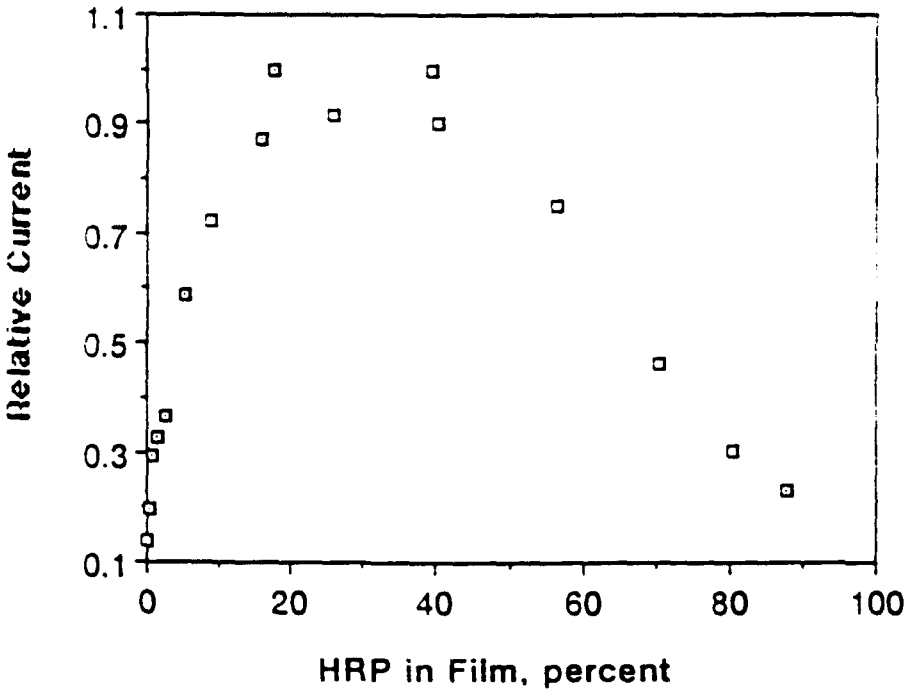
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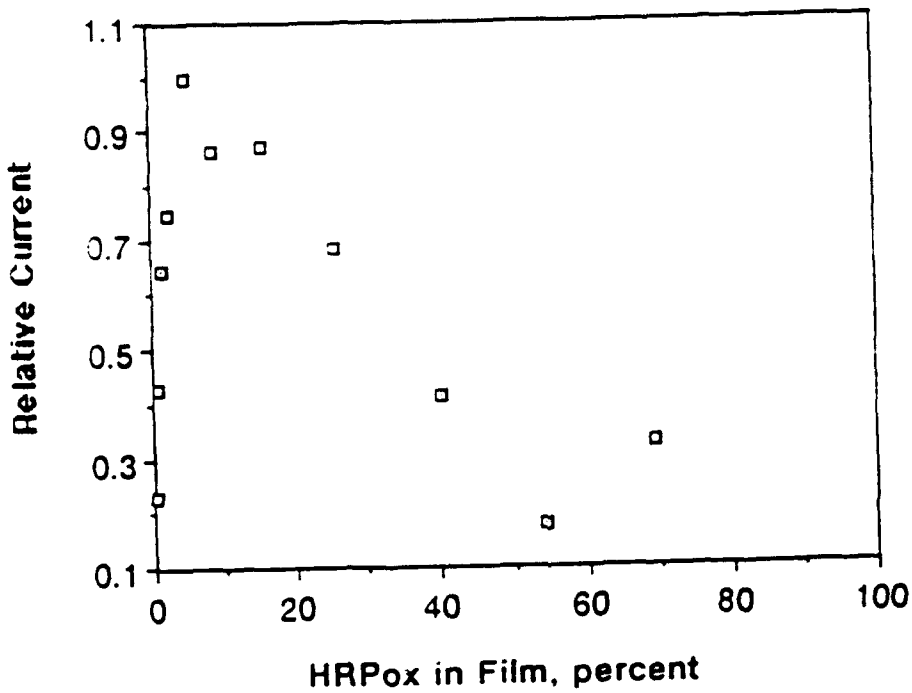
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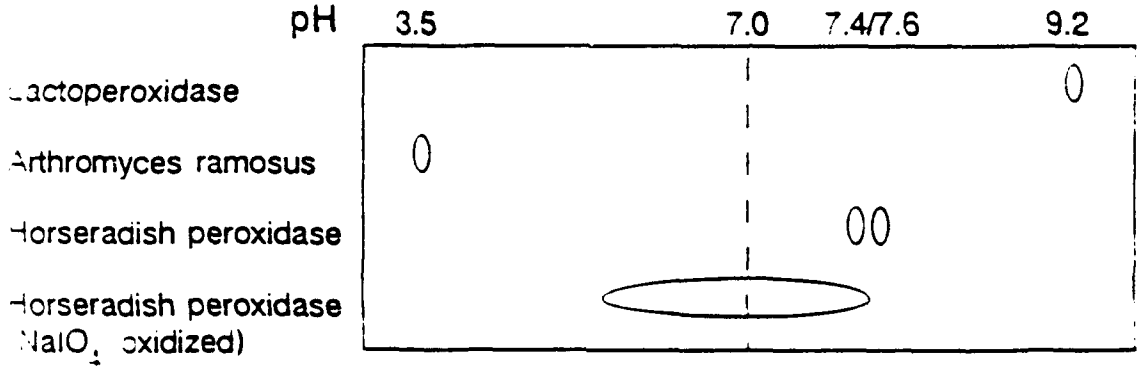
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9.2
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Horseradish peroxidase
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(NaIO4 oxidized)
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